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Carbohydrate esterases of family 2 are 6-*O*-deacetylases

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ABSTRACT

Three acetyl esterases (AcEs) from the saprophytic bacteria *Cellvibrio japonicus* and *Clostridium thermocellum*, members of the carbohydrate esterase (CE) family 2, were tested for their activity against a series of model substrates including partially acetylated gluco-, manno- and xylopyranosides. All three enzymes showed a strong preference for deacetylation of the 6-position in aldohexoses. This regioselectivity is different from that of typical acetylxylan esterases (AcXEs). In aqueous medium saturated with vinyl acetate, the CE-2 enzymes catalyzed transacetylation to the same position, i.e., to the primary hydroxyl group of mono- and disaccharides. Xylose and xylooligosaccharides did not serve as acetyl group acceptors, therefore the CE-2 enzymes appear to be 6-*O*-deacetylases.

1. Introduction

Acetylxylan esterases (AcXEs; EC 3.1.1.72) are common components of the hemicellulolytic and cellulolytic enzyme systems of microorganisms proliferating on plant biomass residues [1]. Their function is to deesterify partially acetylated hardwood 4-*O*-methyl-D-glucuronoxylan and xylans of annual plants. These esterases are found in 8 of 16 known carbohydrate esterase (CE) families (http://www.cazy.org/fam/acc_CE.html, [2]).

Members of the CE-2 family are especially intriguing with respect to the multiple functions required of plant degrading enzyme systems. CE-2 family esterases were first described in *Neocallimastix patriciarum* and were shown to be acetyl esterases (AcEs), which are active on synthetic aryl-esters, with low or no activity against acetylated birchwood xylan [3]. Recently, the crystal structure and biochemical properties of several CE-2 members, both single-module CE-2 enzymes from *Cellvibrio japonicus* (*Cj*CE2A, B), and a component of a modular cellulase *Ct*Cel5C from *Clostridium thermocellum* (*Ct*CE2) were resolved, revealing an α/β hydrolase fold [4]. The esterases *Cj*CE2B and *Ct*CE2 provide a rare example of a catalytic dyad (Ser & His) with stabilization of the His residue, mediated by main chain carbonyl groups. Surprisingly, the *Ct*CE2 module, which is linked to the cellulase *Ct*Cel5C, displays divergent catalytic esterase and non-catalytic carbohydrate binding functions, illustrating a rare example of the "gene sharing" hypothesis, where the introduction of a second functionality into the active site of an enzyme does not compromise the original activity of the biocatalyst [4].

Members of the CE-2 family act as AcEs, releasing acetate from activated artificial substrates, such as 4-nitrophenyl acetate, while some show high deacetylating activity against acetylated konjac glucomannan, compared to acetylated birchwood xylan [4]. It is interesting that in the elegant work of Montanier et al. [4], the positional specificity of the enzymes as potential 6-*O*-deacetylases was not investigated, despite some hints that konjac glucomannan is a linear random copolymer of (1→4) linked β-D-mannose and β-D-glucose with a low degree of acetylation at the C-6 position [5,6]. In the present work, we describe the substrate and positional specificity of three recombinant CE-2 esterases from *C. japonicus* and *C. thermocellum* against a series of model substrates, including partially acetylated gluco-, manno- and xylopyranosides. The enzymes were also successfully tested for their synthetic activity and regioselectivity of acetylation of different biomass-derived

Abbreviations: CE, carbohydrate esterases; AcE, acetyl esterase; AcXE, acetylxylan esterase; 4-NPh–OH, 4-nitrophenol

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carbohydrates. This is the first report that members of CE-2 family show, in both hydrolytic and synthetic functions, a regioselectivity distinct from that of AcXEs and appear to be specialized for the 6-*O*-deacetylation of glucopyranosyl and mannopyranosyl residues.

2. Materials and methods

2.1. Enzyme source

Recombinant *C. japonicus Cj*CE2B (ACE85322.1), *Cj*CE2C (ACE85140.1) and *C. thermocellum Ct*CE2 domain (ABN52032.1) were heterologously expressed in *Escherichia coli* BL21 (DE3) and purified by immobilized metal affinity chromatography, as described previously [4]. β-Xylosidase used in the enzyme-coupled assay of AcEs, was a product of *Aspergillus niger* gene *xlnD*, expressed in recombinant *Saccharomyces cerevisiae* Y294 [7].

2.2. Model compounds and reagents

The three monoacetylated 4-nitrophenyl β-D-xylopyranosides **1–3** were synthesized previously [8]. Methyl 6-*O*-acetyl-β-D-glucopyranoside **4** was prepared according to Lindberg [9]. Compound **5** was prepared by enzymatic acetylation in this work (see below). The three methyl di-*O*-acetyl-β-D-xylopyranosides **6–8** have been described by Kovac and Alföldi [10–12]. 6-*O*-Methyl-D-glucopyranose **15** and its methyl α-glycoside **14** were synthesized according to Bourne et al. [13] and Evtushenko [14], respectively. The model disaccharides related to glucoxylans and xyloglucans **17**, **18** and **20** were prepared according to Petráková et al. [15].

2.3. Substrate specificity reactions

The β-xylosidase-coupled assay, using 2-*O*-, 3-*O*-, or 4-*O*-acetyl 4-nitrophenyl β-D-xylopyranoside (compounds **1–3**, Fig. 1), was conducted at 40 °C in a total volume of 105 μl in 0.1 M sodium phosphate buffer, pH 5.5. Stable 200 mM stock solutions of substrates were prepared in dimethyl sulfoxide and diluted to working concentrations with the preheated phosphate buffer just before the assay. To measure deacetylase activity, the reaction was started by the addition of the CE-2 enzyme solution (2.5 μl) to a mixture of the substrate and β-xylosidase (102.5 μl). The absorbance of the liberated 4-nitrophenol (4-NPh–OH) was monitored at 405 nm against substrate and enzyme blanks, using a SPECTRAmax 250 Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA). The recombinant β-xylosidase, did not liberate 4-NPh–OH from the 4-nitrophenyl β-D-xyloside monoacetates at an appreciable rate within 15 min. Kinetic constants (k_{cat}, K_m) were estimated, using a non-linear regression model (GraFit) that also gives an estimate of the standard error of each parameter [16].

In order to probe the substrate specificity of CE-2 esterases against acetylated D-mannopyranose, methyl β-D-gluco- and β-D-xylopyranosides (compounds **4–8**, Fig. 1), respectively, 10 mM of each substrate was tested at 40 °C in a total volume 105 μl in 0.1 M sodium phosphate buffer, pH 6.0. The reaction was started by the addition of the enzyme solution (5 μl). The β-D-xylopyranosides diacetate solutions were freshly prepared in order to avoid acetyl group migration [8]. Deesterification of compounds was followed qualitatively by TLC and quantitatively by determination of the remaining ester, according to Hestrin [17]. This method is based on the conversion of the ester into hydroxamic acid by a reaction with hydroxylamine under alkaline conditions. The hydroxamic acid is in turn complexed with ferric ion, giving rise to a brownish chromophore measured spectrophotometrically at 540 nm.

The protein concentration (mg ml^{-1}) of the purified CE-2 esterases were estimated spectrophotometrically at 280 nm, using molar extinction coefficient values calculated from their protein sequence.

2.4. Enzymatic transacetylation reactions

Enzymatic acetylation of monosaccharides (D-glucose (Glc), D-mannose (Man), D-galactose (Gal) and D-xylose (Xyl); 40 mg ml^{-1}), their methyl glycosides and their mono-methyl ethers (40 mg ml^{-1}), was carried out in 100 mM 3-(*N*-morpholino)propanesulfonic acid–NaOH buffer pH 6.0 saturated with vinyl acetate, as reported previously [18]. The reactions were initiated by the addition of the enzyme (16 μg), followed by agitation at 30 °C in an Eppendorf Thermomixer Comfort (Eppendorf, Germany), operating at 1400 rpm for 24 h and analyzed by HPLC (monosaccharides) or TLC. All reaction mixtures contained carbohydrate acceptors (100 μl), purified recombinant CE-2 esterases and 20 μl of vinyl acetate. Control samples lacking enzyme preparation or acyl donor were run simultaneously.

2.5. Analytical procedures

Reaction mixtures using compounds **9–22** (Fig. 3) as acetyl acceptors, were analyzed by TLC on aluminium sheets coated with Silica gel 60 (Merck, Germany), in the acetonitrile/water 9:1 (v/v)

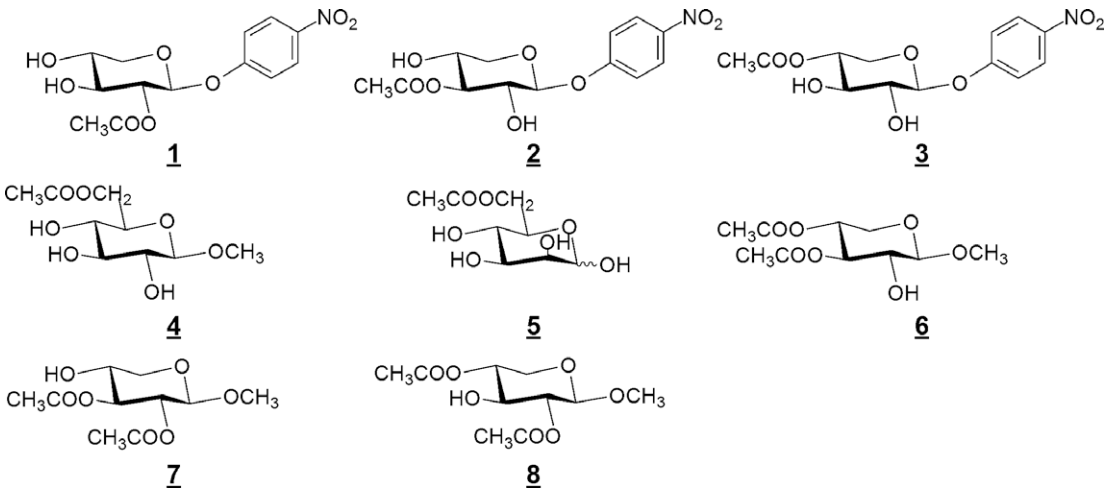

Fig. 1. Chemical structures of model substrates used for probing the hydrolytic specificity of CE-2 esterases, including 4-nitrophenyl β-D-xylopyranoside monoacetates, partially acetylated D-mannopyranose and methyl β-D-gluco- and β-D-xylopyranosides.

Table 1
Kinetic constants for hydrolysis of 4-nitrophenyl β-D-xylopyranoside monoacetates by *Cj*CE2B (A) and *Cj*CE2C (B) from *C. japonicus* and *Ct*CE2 (C) from *C. thermocellum*. Numbers in parentheses are the estimates of the standard errors. n.d., activity not determined.

Substrates	Name	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m
A. *Cj*CE2B				
1	2-*O*-Acetyl 4-nitrophenyl β-D-xylopyranoside	4.42 (0.82)	2496 (332)	565 (129)
2	3-*O*-Acetyl 4-nitrophenyl β-D-xylopyranoside	1.55 (0.38)	126 028 (24 674)	81 361 (25 467)
3	4-*O*-Acetyl 4-nitrophenyl β-D-xylopyranoside	1.24 (0.15)	192 614 (12 122)	155 912 (21 345)
B. *Cj*CE2C				
1	2-*O*-Acetyl 4-nitrophenyl β-D-xylopyranoside	n.d.	n.d.	n.d.
2	3-*O*-Acetyl 4-nitrophenyl β-D-xylopyranoside	n.d.	n.d.	n.d.
3	4-*O*-Acetyl 4-nitrophenyl β-D-xylopyranoside	4.31 (1.06)	14 493 (2637)	3365 (1030)
C. *Ct*CE2				
1	2-*O*-Acetyl 4-nitrophenyl β-D-xylopyranoside	n.d.	n.d.	n.d.
2	3-*O*-Acetyl 4-nitrophenyl β-D-xylopyranoside	n.d.	n.d.	n.d.
3	4-*O*-Acetyl 4-nitrophenyl β-D-xylopyranoside	2.46 (0.42)	79 873 (8662)	32 493 (6567)

solvent system. Sugars were detected on dried chromatograms, using the *N*-(1-naphthyl)ethylenediamine dihydrochloride reagent, according to Bounias [19]. The chromatograms treated with the detection reagent were heated at 110 °C in an oven and cooled prior to analysis.

The acetylation of monosaccharides (Glc, Man, Gal, Xyl) was quantified, using an HPLC (Waters 600E), equipped with a refractometer (Waters 410), on a NH_2-Macherey Nagel 250/4.6 100–5 column. Elution was conducted with a mixture of acetonitrile/water (9:1, v/v) at a flow rate of 1 ml min^{-1}. Yields for the synthesis of the acetylated monosaccharides were calculated from the amount of each individual monosaccharide reacted compared to its initial unreacted concentration. The monosaccharide concentrations were calculated from standard curves, obtained under the same analytical conditions.

2.6. Structure elucidation of acetylated monosaccharides

Proton NMR spectroscopy was performed in CD_3OD with a Bruker DRX 400 spectrometer, equipped with a 5 mm $^1H/^{13}C$ dual inverse broad probe at 400.13 MHz.

Mass spectrometry was done by direct infusion of the reaction products dissolved in 50:50 methanol/water (v/v) with 0.2% formic acid solution, in a Varian 500 MS Quadrupole Iontrap LC/MS. The mass spectra were obtained in positive mode, using the electrospray ionization (ESI) technique. Various parameters, such as capillary voltage, drying gas temperature, drying and nebulizer gas pressure, were adjusted in order to optimize the signal.

3. Results and discussion

3.1. Substrate specificity of CE-2 family AcEs

To probe the function and role of the diverse members of the CE-2 esterase family, which currently contains 38 members (http://www.cazy.org/fam/CE2.html), the biochemical properties of three CE-2 enzymes were assessed. In addition to *Ct*CE2, which corresponds to the C-terminal region of the *C. thermocellum* bifunctional protein *Ct*Cel5C-CE2, we also characterized two *C. japonicus* CE-2 members, *Cj*CE2B and *Cj*CE2C, which were cloned and expressed in *E. coli*, as described previously [4].

In order to probe the positional specificity of CE-2 esterases on the xylopyranose ring, we used the enzyme-coupled assay with three different monoacetylated 4-nitrophenyl β-D-xylopyranosides (Fig. 1; compounds **1**–**3**). As shown in Table 1, all CE-2 tested showed strong preference for the deacetylation of positions 4 and 3. This finding is in contrast to the preference of AcXEs for position 2 [20], but similar to the positional specificity of the AcE

Table 2
Hydrolysis of synthetic substrates by CE-2 AcEs from *C. japonicus* and *C. thermocellum*. Specific activities for partially acetylated D-mannopyranose and methyl β-D-gluco- and β-D-xylopyranosides. Compound **5** was synthesized by *Cj*CE2B.

Substrates	Name	Specific activity (U/mg)		
		*Cj*CE2B	*Cj*CE2A	*Ct*CE2
4	Methyl 6-*O*-acetyl-β-D-glucopyranoside	328	60	183
5	6-*O*-Acetyl-D-mannopyranose	395	3	35
6	Methyl 3,4-*O*-diacetyl-β-D-xylopyranoside	4	3	8
7	Methyl 2,3-*O*-diacetyl-β-D-xylopyranoside	0	0	0
8	Methyl 2,4-*O*-diacetyl-β-D-xylopyranoside	5	2	2

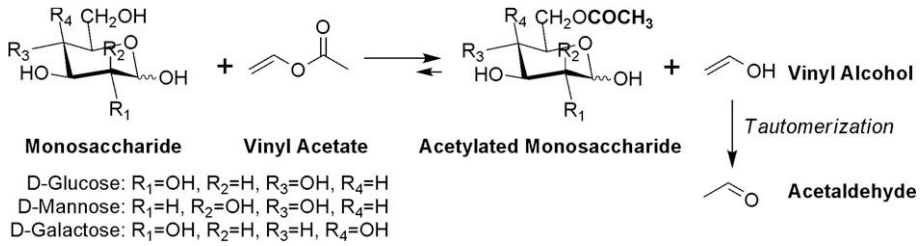

Fig. 2. Transacetylation reaction of monosaccharides with vinyl acetate catalyzed by CE-2 AcEs.

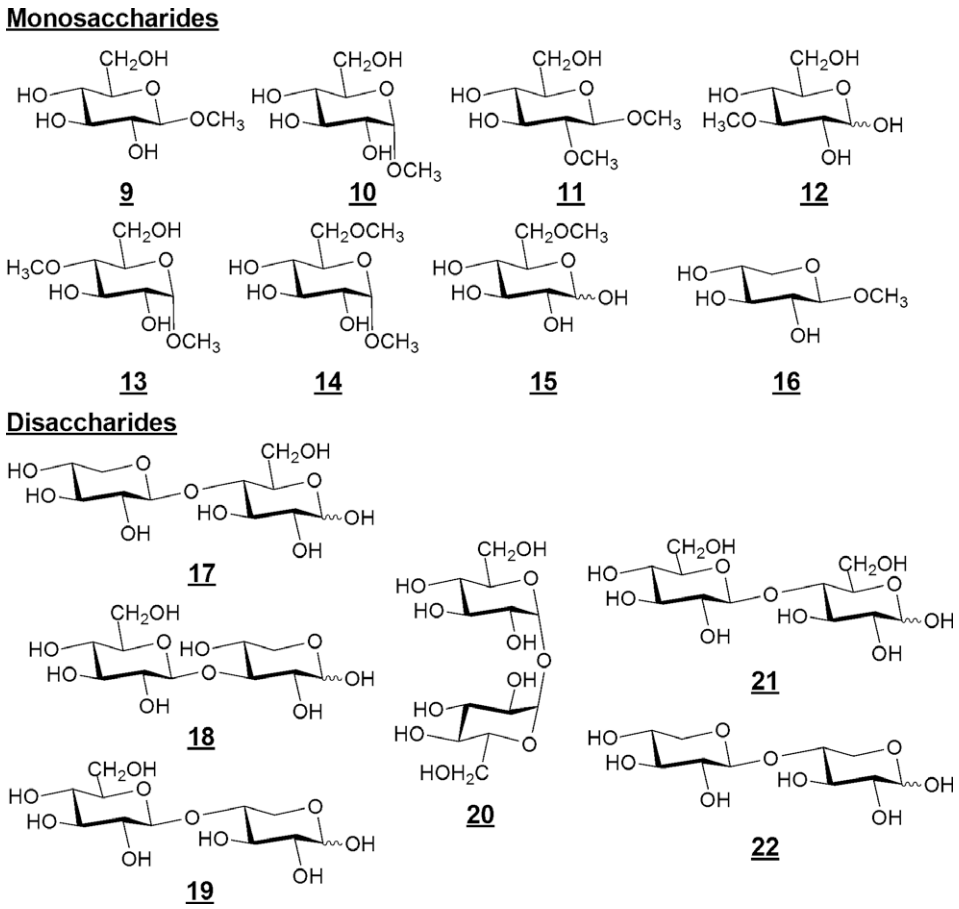

Fig. 3. Chemical structures of model substrates used for probing the synthetic specificity of CE-2 esterases, including various methyl ethers of D-glucose and methyl glycosides of D-glucopyranose and D-xylopyranose.

Table 3
Transacetylation of various monosaccharides with vinyl acetate catalyzed by CE-2 AcEs at 30 °C after 24 h.

Name	Transacetylation conversion (%)		
	*Cj*CE2B	*Cj*CE2A	*Ct*CE2
D-Glucose	31	25	86
D-Mannose	46	26	71
D-Galactose	22	48	89
D-Xylose	0	0	0

from *Hypocrea jecorina*, which is a member of the recently established CE-16 family [21]. Thus, the mode of action of CE-2 and CE-16 AcEs on xylopyranosyl residues could be complementary to the action of AcXEs for the complete removal of acetyl groups during hemicellulose biodegradation.

Recently, CE-2 AcEs and in particular *Cj*CE2B and *Ct*CE2, have been reported to display a significant preference for acetylated konjac glucomannan [4] which, in view of the scarce information on 6-*O*-acetylation of the polysaccharide [5,6], might indicate activity for the deacetylation of gluco- and mannopyranoside residues at 6-position. A series of partially acetylated D-mannopyranose and methyl β-D-gluco- and β-D-xylopyranosides (compounds **4**–**8**) were tested as substrates for probing the catalytic specificity of the CE-2 esterases. As shown in Table 2, all CE-2 esterases tested showed strong preference for deacetylation of methyl 6-*O*-acetyl-β-D-glucopyranoside and 6-*O*-acetyl-D-mannose, compared to their activity against diacetylated xylopyranosides. The highest specific activity for 6-*O*-acetylated substrates was exhibited by *Cj*CE2B AcE, which might explain the high deacetylating activity reported for this enzyme against konjac glucomannan [4]. The resistance of methyl 2,3-di-*O*-acetyl-β-D-xylopyranoside to hydrolysis is more or less in consonance with the behaviour of the enzymes on xylopyranoside monoacetates (Table 1). The conclusion from the above results is that all three CE-2 enzymes show clear preference for deacetylation of the primary hydroxyl group in gluco- and mannopyranosyl residues.

3.2. Synthetic potential and specificity of CE-2 AcEs

In order to evaluate the synthetic potential of CE-2 AcEs, we carried out transacetylation reactions in two-phase system, composed of an aqueous solution of the acceptor and vinyl acetate as the acetyl group donor (Fig. 2; [18,22]). As shown in Table 3, all CE2 tested were able to catalyze the acetylation of Glu, Man and Gal with the *Ct*CE2 esterase showing the highest conversion yields. On the other hand, none of the esterases were able to acetylate Xyl, a reaction that was carried out successfully by the CE-16 AcE from *H. jecorina* in the same reaction system and conditions [18,22], indicating a divergence with respect to the synthetic mode of action between AcEs belonging to different CE families. The products of Glc and Man acetylation by *Cj*CE2B were isolated from the reaction mixtures by chromatography on Silica gel column. Monoacetylation of both hexoses was demonstrated by ESI MS operating in the positive ion mode, resulting in a singly charged adduct ion $[M+Na]^+$ at *m/z* 245. The presence of the acetyl group at position 6 was confirmed by ^1H NMR (Table 4).

Table 4
^{1}H NMR data of acetylated Glc and Man. Chemical shifts (δ) in ppm.

	H1[a]	H2–H5	H6	CH_3COO-
6-*O*-Acetyl-D-glucopyranose	5.10 (d, *J* = 3.56 Hz, 0.1H), 4.50 (d, *J* = 7.74 Hz, 0.9H)	3.52–3.14 (m, 4H)	4.42 (bs, 0.4H), 4.39 (bs, 0.6H), 4.20 (d, *J* = 6.0 Hz, 0.6H), 4.17 (d, *J* = 6.0 Hz, 0.4H)	2.07 (s, 3H)
6-*O*-Acetyl-D-mannopyranose	5.07 (bs, 0.8H), 4.78 (bs, 0.2H)	3.62–3.96 (m, 4H)	4.40 (d, *J* = 1.9 Hz 0.4H), 4.37 (d, *J* = 1.9 Hz 0.6H), 4.25 (d, *J* = 6.2 Hz, 0.6H), 4.22 (d, *J* = 6.2 Hz, 0.4H)	2.07 (s, 3H)

[a] Two peaks due to mixture of anomers.

Table 5
Semiquantitative determination of the rate of acetylation of various methyl ethers of D-glucose and methyl glycosides of D-glucopyranose and D-xylopyranose by *Cj*CE2B and *Cj*CE2A from *C. japonicus* and *Ct*CE2 from *C. thermocellum* in aqueous solution saturated with vinyl acetate. Rate of acetylation: excellent acetyl group acceptors, +++; weaker acetyl group acceptors, ++ and +; no acceptor, –.

Substrates	Name	Rate of acetylation		
		*Cj*CE2B	*Cj*CE2A	*Ct*CE2
9	Methyl β-D-glucopyranoside	+++	++	+
10	Methyl α-D-glucopyranoside	+++	+	+++
11	Methyl 2-*O*-methyl-β-D-glucopyranoside	+++	++	+++
12	3-*O*-Methyl D-glucopyranose	+++	++	++
13	Methyl 4-*O*-methyl-α-D-glucopyranoside	+++	+	+++
14	Methyl 6-*O*-methyl-α-D-glucopyranoside	–	–	–
15	6-*O*-Methyl D-glucopyranose	–	–	–
16	Methyl β-D-xylopyranoside	–	–	–
17	Xylopyranosyl β-(1,4)-glucopyranose	++	–	+
18	Glucopyranosyl β-(1,3)-xylopyranose	+++	+++	+++
19	Glucopyranosyl β-(1,4)-xylopyranose	+++	+	++
20	α-D-Glucopyranosyl α-D-glucopyranoside (α,α-trehalose)	+	–	–
21	Cellobiose	+++	+++	+++
22	Xylobiose	–	–	–

The synthetic reaction of CE-2 recombinant AcEs was also examined, using various mono-methyl ethers of D-glucose and methyl D-glucopyranoside and also methyl β-D-xylopyranoside (compounds **9**–**16**; Fig. 3) as acetylation acceptors. As shown in Table 5, the three CE-2 esterases acetylated both anomeric forms of methyl D-glucopyranoside (compounds **9** and **10**) with *Ct*CE2 esterase showing a slight preference for the acetylation of the α-anomer. The results in Table 5 show that CE-2 esterases acetylated all compounds, with exception of 6-*O*-protected D-glucose or D-glucopyranoside, indicating a strong regioselectivity for acetylation of the primary hydroxyl group. This finding is in accordance with their hydrolytic specificity profile. The 6-*O*-acetylation also appears to be the predominant reaction with disaccharides as acetyl group acceptors (compounds **17**–**22**, Fig. 3, Table 5). Xylobiose did not serve as an acceptor. Of three mixed-type disaccharides containing glucose and xylose (compounds **17**–**19**), a higher degree of acetylation was observed with dimmers having a glucopyranosyl residue at the non-reducing end. These results indicate that the enzymes can differentiate between reducing and non-reducing aldohexose residues in oligosaccharides as their target. This is further justified by the monoacetylation of cellobiose compared to trehalose. An explanation of this phenomenon, which might be associated with sugar binding subsites in the substrate binding site of the CE-2 enzymes, would require a study that would include larger oligosaccharides and eventually polysaccharides as acetyl group acceptors.

A unique property of the esterase enzymes studied in the present paper is catalysis of acetyl transfer to alcohols in water, in particular the efficient regioselective 6-*O*-acetylation of aldohexoses in aqueous medium saturated with vinyl acetate. This reaction may be considered to achieve another hitherto unattainable goal of biocatalysis with important future applications in carbohydrate and material chemistry. Elucidation of the physiological role of CE2 esterases requires further studies that include identification of the natural substrate for the enzymes.

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